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Markers of Ovarian Cancer Using a Glycoprotein/Antibody Array

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14. ABSTRACT

Profiling of the glycan structures that differ between the serum of serous ovarian cancer and benign conditions have been performed using lectin arrays for biomarker studies. These arrays have used 16 different lectins which respond to specific glycan structural moieties of glycoproteins in patient serum. It has been found that there are distinct changes in the level of fucosylation between cancer and benign using LCA, AAL and UEA lectins which detect both core and outer arm glycosylation. Some difference was also observed for SNA which detects 2,6 sialylation. Using these lectins we have been able to extract the glycoproteins from patient serum and are evaluating the differentially expressed glycoproteins based on mass spec and glycoarray technologies. We have identified 10-12 glycoproteins which show distinct changes in expression between benign conditions and stage 3c serous ovarian cancer. These glycoproteins will be used for further validation studies in future work.

15. SUBJECT TERMS

Glycoproteins, Biomarkers, lectins, microarrays

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Introduction: We are developing a lectin/antibody assay for biomarker discovery and validation in ovarian cancer(1-3). The novel concept here as opposed to most current marker studies is that the marker depends upon structural changes of glycosylation on a specific protein rather than on the abundance of the protein itself. Many of the markers may be relatively high abundance glycoproteins found in serum, but it is the glycan group whose structure changes in a well regulated manner during cancer progression that will be monitored rather than the abundance of the glycoprotein itself. These glycoprotein markers may be present in many cancers but will be shown to change in a very specific manner for each different type of cancer and stage of cancer and also for other benign conditions.

Description of Progress:
During the current progress period we have been working on identifying changes in ovarian cancer serum using a lectin based approach. According to the statement of work this involves SOW 1a) Using a lectin array with 16 lectins to determine which lectins show the greatest overall difference between early stage ovarian cancer serum response and normal controls and SOW 1b.) Choose lectins based on the lectin array and use for extracting specific N-linked glycoproteins from patient serum for each patient.

In order to find the largest differences between ovarian cancer and benign conditions we chose to screen for specific lectins which can distinguish ovarian cancer stage IIIC – untreated serum samples from benign samples. The initial set of serum samples consisted of 12 benign and 22 ovarian cancer stage IIIC – untreated. The Twelve most high-abundance proteins were depleted from 250 µL serum. The depleted samples were concentrated using a YM-3 centrifugal device. Subsequently, protein concentration was measure by Bradford assay kit.

Sixteen kinds of lectins were printed on nitrocellulose coated glass slides using a piezoelectric non-contact printer to form a lectin array(4-5). The concentration of lectins is 1 mg/ml and each lectin was printed in triplicate. After printing, the slides were blocked and washed three times. Ten micrograms of serum proteins were reduced by 5 mM TCEP for 30 min. The reduced proteins were labeled by EZ-link iodoacetyl-LC-biotin for 1.5 hr. Un-reacted labeling reagent was removed by a desalting column. The labeled serum samples were hybridized with slides for 1 hr followed by incubation with steptavidinylated fluorescent dye for 1 hr . The signal intensity was detected by a microarray fluorescent scanner.

After Students T-test analysis, 4 lectins showed a significant response difference between cancer and benign. They include LCA, UEA-I, AAL, and SNA. The former 3 lectins have glycosylation binding specificity toward fucosylation (Fig. 1) while the latter one prefers to capture the Neu5Acα2-6Gal(NAc)-R structure (Fig. 2). The response of these 4 lectins in benign samples is higher than in cancer samples. Considering that in our work on other cancers the fucosylation level in the cancer serum was found to be different from benign samples(6), we will focus on the fucosylated protein identification and quantification in the future experiments.

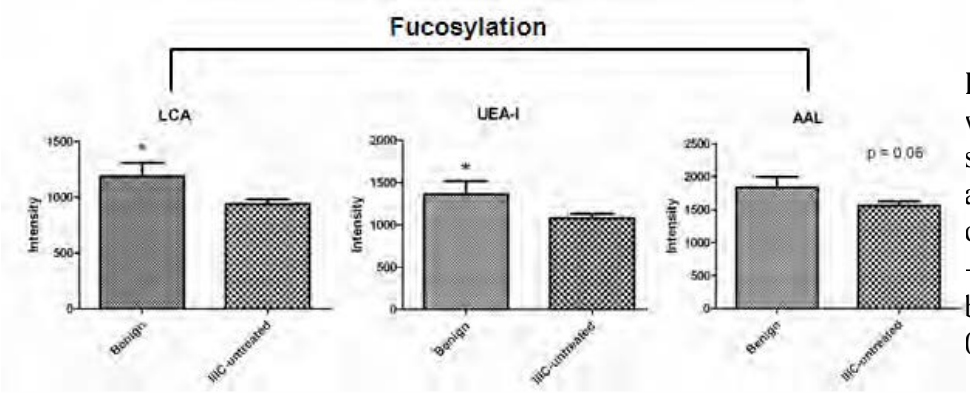

Figure 1. Three lectins with fucosylation-binding specificity, LCA, UEA-I, and AAL, can differentiate ovarian cancer stage IIIC – untreated samples from benign samples (*, $p < 0.05$).

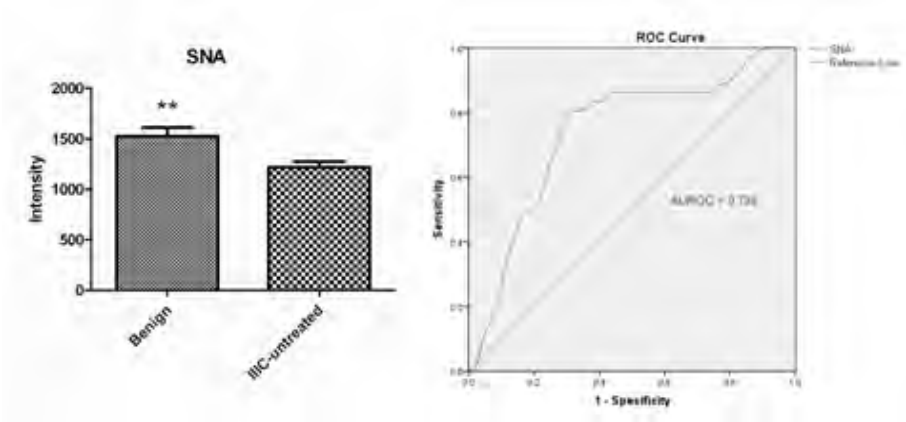

Figure 2 . SNA with Neu5Acα2-6Gal(NAc)-R binding specificity can differentiate ovarian cancer stage I IIC – untreated samples from benign samples (**, $p < 0.01$).

SOW 1(c-d.) involves making glycoarrays of the glycoproteins extracted based on fucosylation for different stages of cancer and hybridizing them to different lectins to study the response.

In addition to making the glycoarrays, we have used an alternative method to accomplish the same task. This involves extraction of the fucosylated glycoproteins using a lectin column and isotopic labeling of the proteins. The proteins are then digested and analyzed by LC-MS/MS. This method allows us to quantitatively analyze differences in fucosylation changes between cancer and benign. We have used LCA, UEA and SNA to each separately extract the fucosylated/ sialylated proteins and performed quantitative isotopic proteomics for the 22 cancer and 12 benign samples. The data is currently being analyzed to search for the potential markers that fall within significance. This data is being compared to the method using a lectin column to extract the glycoproteins and to print arrays of these proteins to hybridize against different lectins. The two methods should provide complementary data and is in progress. In Table 1 below is shown the top 10 candidates which have been selected based on our current work and which lectin was used to detect a differential response between benign and cancer stage 3. There are additional candidates, but we have selected the ones in Table 1 as the most significant in our analysis. There are several proteins that are related to ovarian cancer and will be selected for further validation at some point. CBG is the most interesting given the potential role of steroids in tumorigenesis(7). We have recently obtained several stage I and II serous ovarian cancers which we will incorporate into this work flow. This work is currently in month 9 and is scheduled to go on until month 18.

Table 1: Candidate Markers for Ovarian Cancer

No.	Accesstion No.	Protein Name	Protein Description	Lectin Affinity	*p* value	Function
1	P08185	CBG	Corticosteroid-binding globulin	LCA	0	Major transport protein for glucocorticoids and progestins in the blood
2	P04196	HRG	Histidine-rich glycoprotein	LCA	0.05	It can inhibit rosette formation and is known to interact with heparin, throm
3	P00751	CFAB	Complement factor B	LCA	0.05	Part of complement system
4	Q96PD5	PGRP2	N-acetylmuramoyl-L-alanine amidase	UEA-I	0.02	May play a scavenger role by digesting biologically active peptidoglycan into
5	P05543	THBG	Thyroxine-binding globulin	UEA-I	0.01	Major thyroid hormone transport protein in serum
6	P08603-2	CFAH	Isoform FHL-1 of Complement factor H	SNA	0.02	Part of complement system
7	P02750	A2GL	Leucine-rich alpha-2-glycoprotein	SNA	0.02	It is expressed during granulocyte differentiation
8	P02774	VTDB	Vitamin D-binding protein	SNA	0.02	In plasma, it carries the vitamin D sterols and prevents polymerization of acti
9	P02790	HEMO	Hemopexin	SNA	0.04	Binds heme and transports it to the liver for breakdown and iron recovery, af
10	P10909	CLUS	Clusterin	SNA	0.05	Not yet clear. It is known to be expressed in a variety of tissues and it seems

Key Research Accomplishments:

- significant differences in the level of core and arm fucosylation between ovarian cancer and benign samples
- 10-12 candidates that show differences between benign and ovarian cancer samples to be used for further validation

Reportable Outcomes:

Postdoctoral Trainee: Xiaolei Xie received a staff scientist position at Caprion Pharmaceuticals in Menlo Park, CA based on this work.

Conclusion:

There are significant changes in fucosylation between ovarian cancer samples and benign conditions which can be used with lectin columns to extract the proteins responsible for these changes. These proteins can then be further profiled as potential markers for ovarian cancer. We have identified 10-12 potential markers of ovarian cancer that may be candidates for further validation. Some of these proteins are known to be related to processes that occur in the progression of cancer. In current work we are incorporating a limited number of stage I and II serous ovarian cancers into our workflow. In the coming year, the markers identified will undergo an antibody/lectin sandwich assay validation for the top markers identified in our discovery set as per the SOW. The markers that successfully pass initial validation would need to undergo further validation but could be developed into a blood test for ovarian serous carcinoma.

References:

1. Zhao J, Patwa TH, Qiu W, Shedden K, Hinderer R, Misek DE, Anderson MA, Simeone DM, and Lubman DM. Glycoprotein microarrays with multi-lectin detection: unique lectin binding patterns as a tool for classifying normal, chronic pancreatitis and pancreatic cancer sera. *J Proteome Res, 2007,* 6, 1864-1874.
2. Li C, Simeone DM, Brenner DE, Anderson MA, Shedden KA, Ruffin MT, et al. Pancreatic Cancer Serum Detection Using a Lectin/Glyco-Antibody Array Method. J Proteome Res. 2009 , 6, 8(2), 483-92.
3. Zhao, J ., Simeone, D .M., Heidt, D ., Anderson, M .A., Lubman, D.M., Comparative Serum Glycoproteomics Using Lectin Selected Sialic Acid Glycoproteins with Mass Spectrometric Analysis: Application to Pancreatic Cancer Serum, J. Proteome Res. 2006, 5, 1792-1802.
4. Zheng T, Peelen D, Smith LM., Lectin arrays for profiling cell surface carbohydrate expression. J Am Chem Soc 2005, 127, 9982-9983.
5. Wearne KA, Winter HC, O'Shea K, Goldstein IJ., Use of lectins for probing differentiated human embryonic stem cells for carbohydrates, Glycobiology 2006, 16, 981-990.
6. Y. Liu, J. He, C. Li, R. Benitez, S. Fu, J. Marrero, D.M. Lubman, Identification and Confirmation of Biomarkers Using an Integrated Platform for Quantitative Analysis of Glycoproteins and Their Glycosylations, Journal of Proteome Research, 2010, 9(2), 798-805.
7. Misao, R ., Iwagaki, S ., Sun, W.S., Fujimoto, J ., Tamaya, T., Levels of Corticosteroid-Binding Globulin mRNA in Human Ovarian Cancers, Tumor Biology 1999, 20, 263-269.